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PRINCIPAL INVESTIGATOR: Lee Ratner, M.D., Ph.D.

CONTRACTING ORGANIZATION: Washington University
School of Medicine
Medical Campus Research Office
724 South Euclid Avenue
St. Louis, Missouri 63110

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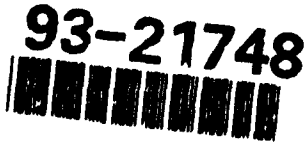

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Medical Campus Research Office
724 South Euclid Avenue
St. Louis, Missouri 63110

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The natural history of HIV-1 infection is characterized by an acute phase of illness within the first few months after infection, a chronic and clinically indolent phase of disease lasting a few months to more than ten years, and a late stage of illness characterized as AIDS with opportunistic infections and malignancies. Progression from the chronic asymptomatic phase of disease to AIDS is characterized by a continual decline in the number of CD4 + Lymphocytes, an increase in the level of viremia, an increase in the number of HIV-1 DNA positive lymphocytes, and an increase in the number of infected cells that express viral gene products. The increase in virus gene expression associated with disease progression is likely critical to the pathogenesis of HIV-1. Increased virus expression may result from changes in host immune regulation and virus-mediated regulatory influences. The goal of the current study was to examine the influence of viral regulatory influences in a cross-sectional cohort of patients at different stages of disease.

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SUMMARY

Reverse transcriptase - polymerase chain amplification reactions (RT-PCR) were used to identify transcripts for HIV-1 structural and regulatory proteins in peripheral blood mononuclear cells of a cohort of 53 patients. At least one set of PCR primers was capable of detecting HIV-1 transcripts in 93% of patients. Unspliced *gag-pol* transcripts were detected with *gag* or *pol* primer sets in 60% and 64% of samples, respectively. A significant correlation was noted between transcript identification with the *gag* primer set and the percentage of CD4 positive lymphocytes in the blood sample. Single-spliced *env* transcripts were identified in 43% of individuals. Multiple-spliced *tat* or *nef* transcripts were detected in 7.5% and 53% of individuals, respectively. For a subset of samples, the level of *env* or *nef* transcripts was assessed, but no correlation was noted with patients' immunological status. These findings indicate that viral transcripts are expressed throughout the course of HIV-1 infection.

FOREWORD

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(5) INTRODUCTION

The natural history of HIV-1 infection is characterized by an acute phase of illness within the first few months after infection, a chronic and clinically indolent phase of disease lasting a few months to more than ten years, and a late stage of illness characterized as AIDS with opportunistic infections and malignancies.[1] Progression from the chronic asymptomatic phase of disease to AIDS is characterized by a continual decline in the number of CD4+ lymphocytes, an increase in the level of viremia,[2,3] an increase in the number of HIV-1 DNA positive lymphocytes,[4,5] and an increase in the number of infected cells that express viral gene products.[6,7] The increase in virus gene expression associated with disease progression is likely critical to the pathogenesis of HIV-1.[1] Increased virus expression may result from changes in host immune regulation and virus-mediated regulatory influences. The goal of the current study was to examine the influence of viral regulatory influences in a cross-sectional cohort of patients at different stages of disease.

The HIV-1 genome includes overlapping reading frames for structural genes (*gag, pol, env*) and regulatory genes (*tat, rev, vif, vpr, vpu, nef*) (Fig. 1A).[8] Structural genes encode proteins found in the virus particle which are important for the assembly and enzymatic functions of the virus. Regulatory genes encode proteins which are generally not found in the virus particle (with the exception of *vpr*) and which are thought to be important for virus replication in the host cell. Two regulatory gene products modulate virus transcription, and were therefore chosen as a major focus of the current study. These include Tat, which is a potent positive effector of virus gene expression[9] and Nef, a weak negative modulator of virus RNA synthesis.[10]

Expression of the HIV-1 genome is mediated by three classes of viral mRNAs: unspliced (9.0 kb), single spliced (4.5 kb), and multiple-spliced (1.8-2.2 kb) transcripts (Fig. 1B-D).[11] Structural genes are encoded by the first two classes of transcripts, with the unspliced mRNA producing *gag* and *pol* proteins and single spliced mRNA producing *env* proteins (as well as *vif, vpr,* and *vpu* products).[12] Regulatory genes (with the exception of *vif, vpr,* and *vpu*) are expressed from multiple-spliced mRNAs. The single-spliced *vpu-env* mRNA utilizes splice donor and splice acceptor sites at nucleotides 287 and 5557 (Fig 1C)[13] (nucleotide positions according to ref. 14). The multiple spliced mRNAs all utilize splice donors at nucleotides 287 and 5625, and a splice acceptor at nucleotide 7956 (Fig. 1D). Utilization of a particular splice acceptor site in the central portion of the genome distinguishes *nef* mRNAs (splice acceptor at nucleotide 5557) from *tat* mRNAs (splice acceptor at nucleotide 5357), and from other regulatory gene transcripts. Additional exons in the central portion of the genome are utilized in regulatory gene transcripts.[13].

The current study has utilized these features of splicing to develop RT-PCR assays capable of distinguishing transcripts for individual HIV-1 structural or regulatory genes. Primers complementary to sequences in different exons were utilized to amplify spliced but not unspliced transcripts (Fig 1C and D). The single-spliced *env* mRNA is distinguished from multiply-spliced regulatory gene mRNAs by using a downstream primer (Senv) complementary to a sequence present in the *env* mRNA, but removed by splicing from regulatory gene mRNAs (Fig. 1C). Different regulatory gene transcripts are distinguished from one another by the use of primers complementary to the sequence spanning the splice acceptor site unique for the particular transcript (S4B at nucleotide 5357 for *tat* mRNA and S7B at nucleotide 5557 for *nef* mRNA).

In the current study, we have used nested sets of primers to perform PCR on RNA extracted from patient peripheral blood mononuclear cells (PBMC) at various stages of disease, as characterized by the number of CD4+ lymphocytes. We have identified structural, as well as regulatory polyA+ RNA transcripts in patients with a wide range of CD4 cell counts. We demonstrated active transcription of mRNA from several regions of the HIV-1 genome in patients who have no symptomatic disease as well as those who have AIDS.

(6) BODY

MATERIALS AND METHODS

Patients

Blood was drawn from 53 HIV-1 seropositive patients presenting to the Washington University AIDS Clinical Trials Unit (ACTU) between October, 1991 and May, 1992. Most patients were being screened for entry onto ACTU protocols and 42 patients had not previously received an antiretroviral drug (Table 1). All patients gave informed consent.

Cells

Peripheral blood mononuclear cells (PBMC) were purified by ficoll-hypaque (LSM, Organon Teknika Corp., Durham, N.C.) gradient centrifugation from heparinized venous blood of patients. Separated cells were washed twice with phosphate buffered saline (PBS) and aliquots were suspended in freeze medium (10% DMSO and 40% fetal calf serum in RPMI medium) and viably frozen at -75° C.

TABLE I : IMMUNOLOGIC MEASUREMENTS AND PCR RESULTS ON PATIENT COHORT

Patient #	Immunologic CD4+ #	CD4 %	CD8 #	CD8 %	T4/T8	AVT*	PCR Results gag	Pol	env genomic	env spl.	tat	Nef	Nef-Seq
1	0	0	223	62	0.0	YES	+	+	+	-	-	-	-
2	0	0	529	55	0.0	YES	+	+	+	+	-	-	-
3	6	7	37	41	.16	YES	+	+	+	-	-	-	-
4	8	1	424	51	.02	YES	+	+	+	+	-	+	-
5	8	1	178	22	.04	YES	+	+	+	+	-	-	-
6	10	5	119	57	.08		+	+	+	-	-	-	-
7	23	2	897	79	.03		-	+	+	+	-	-	+
8	24	2	722	59	.03	YES	+	-	-	-	-	-	-
9	32	5	486	76	.07	YES	+	+	+	-	-	+	-
10	62	4	1287	83	.05		+	+	+	-	+	+	-
11	86	8	713	66	.12		+	-	-	-	-	-	-
12	105	5	990	47	.11	YES	+	-	-	-	-	+	-
13	137	6	1938	85	.07		-	-	-	-	-	+	+
14	177	12	854	58	.21	YES	-	+	+	-	-	+	-
15	185	19	614	63	.30		-	-	+	+	-	+	+
16	191	11	1180	68	.16		+	+	+	+	-	-	+
17	195	21	492	53	.40		+	+	+	-	+	+	-
18	255	16	894	56	.29		-	-	-	+	-	+	+
19	259	16	875	54	.30		+	+	+	-	-	+	-
20	281	15	1161	62	.24		+	+	+	+	-	+	-
21	297	25	654	55	.45		-	+	+	-	-	-	-
22	306	29	444	42	.69		-	-	+	+	-	-	-
23	333	17	1420	58	.29		-	+	-	+	-	+	-
24	336	28	576	43	.65		+	+	-	-	-	+	-
25	358	23	935	60	.38		+	+	+	-	-	+	-
26	365	28	770	59	.47		+	-	+	+	-	+	+
27	370	22	959	57	.39		+	+	+	+	-	-	-
28	370	14	1614	61	.23		+	+	+	+	-	-	-
29	383	25	826	54	.46		-	-	-	-	-	-	-
30	390	29	685	51	.57		-	-	-	-	-	+	-
31	392	30	626	48	.63		-	-	-	+	-	-	-
32	404	29	558	40	.72		-	+	-	-	-	+	-
33	408	17	1392	58	.29	YES	+	+	+	+	+	+	-
34	410	19	1274	59	.32		+	+	+	+	-	+	-
35	421	20	1221	58	.34		+	+	+	-	-	+	-
36	424	16	1962	74	.22		-	-	+	+	-	-	-
37	426	36	463	39	.92		+	+	-	-	-	-	-
38	434	29	763	51	.57		+	+	+	+	-	+	-
39	454	22	1115	54	.41		-	-	-	-	-	-	-
40	460	31	728	49	.63		+	-	-	-	-	-	-
41	490	28	998	57	.49		+	+	-	+	-	+	+
42	535	25	1214	59	.44		+	+	+	-	-	+	+
43	560	32	980	56	.57		-	-	-	+	-	-	-
44	564	18	1942	62	.29		+	+	-	-	-	+	-
45	569	19	2065	69	.28		-	+	+	+	-	-	-
46	587	26	1331	59	.44		-	-	-	-	-	-	-
47	599	29	1178	57	.51		+	+	+	-	+	+	-
48	605	36	739	44	.82		+	+	+	+	-	+	-
49	647	33	902	46	.72		+	-	-	-	-	-	-
50	686	26	1399	53	.49	YES	-	+	+	-	-	+	-
51	822	31	1405	53	.59		-	+	-	+	-	+	+
52	902	52	520	30	1.73		-	-	-	-	-	-	-
53	1280	49	706	27	1.81		-	-	+	-	-	-	-

*AVT = antiviral therapy; yes, pts were known to have been on therapy within the previous 6 months; blanks indicate pt had not been on therapy.

Purification and Quantitation of Polyadenylated RNA

Samples of patient cells, frozen as described above, were thawed, and the number of viable cells counted in the presence of trypan blue (>95% viable). Aliquots of 5 x 10^6 cells were used for isolation of poly A^+ RNA according to the Micro-Fast Track procedure (Invitrogen, San Diego, CA.). The purified polyA + RNA was treated with RNase-free DNase I (Boehringer Mannheim, Gonbt, Germany, 40 units) at 37° C for 10 min in the presence of 1 ug of carrier tRNA, 10 mM $MgCl_2$, 0.1 mM dithiotheritol and 200 U RNasin (Promega, Madison, WI). Each reaction was stopped by addition of 4 ul of 0.5 M EDTA and 4 ul of 10% SDS. DNase-treated polyA + RNA was extracted once with phenol:chloroform:isoamyl alcohol (100:96:4) and the aqueous phase was precipitated by the addition of glycogen carrier (10 ul), 30 ul of 2 M sodium acetate and 3 volumes of cold ethanol and kept at -70° C overnight. The precipitate was collected by centrifugation, dried at room temperature and dissolved in elution buffer.

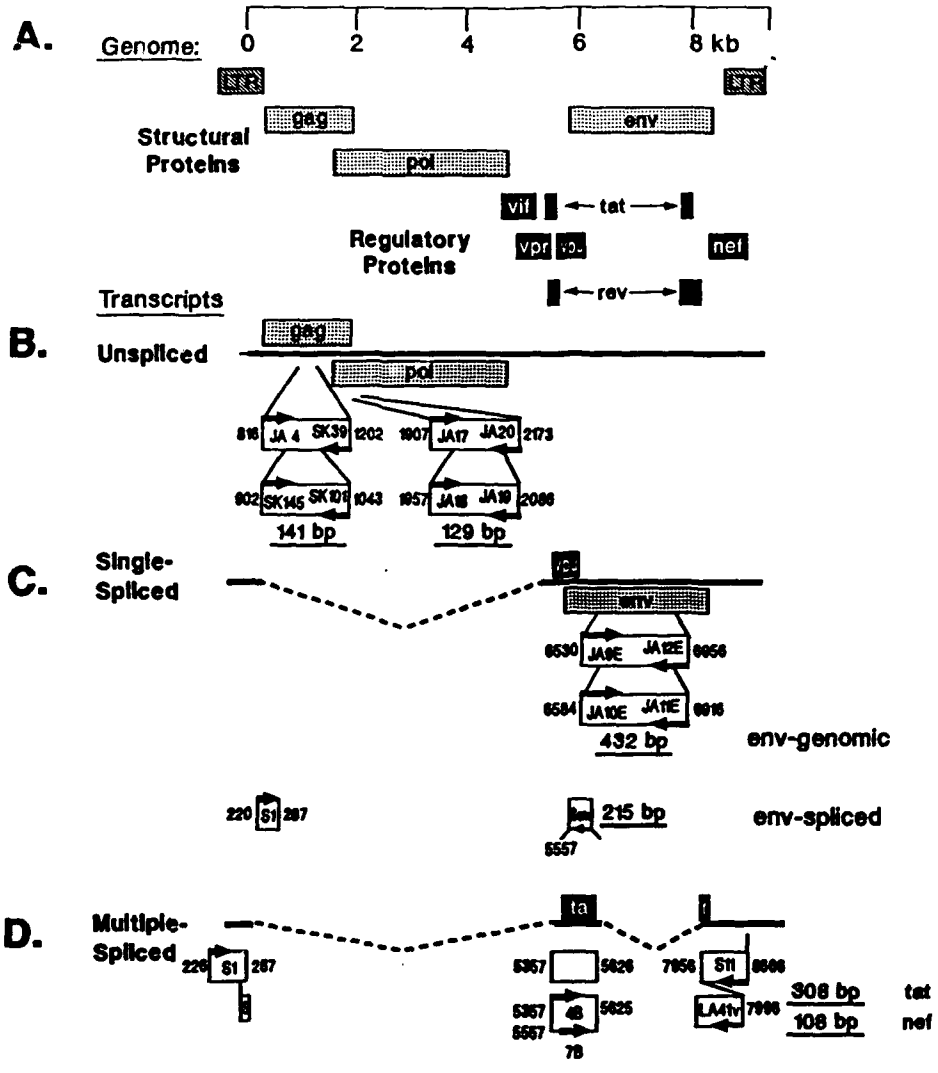
Quantitation of polyA + RNA was accomplished by measuring the incorporation of oligo-dT-directed incorporation in the presence of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) of ^{3}H-dCTP into DE81-retainable material, using purified globin mRNA (Gibco/BRL/Life Technologies, Gaithersburg, MD) as a standard. A 2 ul sample of each purified RNA from above was denatured at 65 C for 3 min and then added to a reaction mixture (final volume 20 ul) containing the following: 80 U RNasin, 0.02 OD of oligo $p(dT)_{12-18}$, 0.4 mM each of dATP, dGTP and dTTP, 5 uCi ^{3}H-dCTP (28 Ci/mmole; ICN Radiochemicals, Irvine, CA), 50 mM Tris-Cl (pH 8.3), 30 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol and 20 U of M-MuLV RT (Gibco/BRL, Gaithersburg, MD). The reaction was incubated at 37° C for 1 hr and stopped by the addition of 4 volumes of 10 mM Tris-Cl (pH 7.5) containing 1 mM EDTA. Duplicate 50 ul samples were spotted onto DE81 filter paper discs and dried. Filters were washed 5 times in 5% sodium phosphate dibasic, once in 95% ethanol, dried and counted in a liquid scintillation counter. A standard curve using 50 pg to 2.5 ng of purified globin mRNA was run with each assay and was used to calculate RNA concentrations of patient samples. Samples that did not fall within the linear portion of the standard curve were diluted appropriately and the assay repeated.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The RT reaction was performed using 1.5 x 10^5 cell equivalents of RNA in a final volume of 20 ul containing the following: 1 mM each of dATP, dCTP, dGTP and dTTP, 6.5 mM $MgCl_2$, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 20 U RNasin, 2.5 uM random primers (Gibco/BRL) and 2.5 U of M-MuLV RT (Gibco/BRL). The reaction was incubated at room temperature for 10 min and then at 42° C for 35 min and was terminated by heating at 99 C for 5 min.

FIGURE LEGEND

Fig. 1. HIV-1 RNA RT-PCR Analysis. A) The schematic drawing of the HIV-1 genome is shown with the nucleotide positions relative to the RNA initiation site indicated above. Genes encoding structural proteins and regulatory proteins are indicated separately. B) The unspliced transcript which encodes *gag* and *pol* proteins is indicated as well as the positions of the nested primer sets in each gene. Arrows indicate positions of the primers, and the designation for each primer is shown in the box above or below the arrow. Nucleotide positions of the primers are indicated outside the boxes. The predicted sizes of the products of the nested PCR reactions are underlined and indicated below the boxes. In the case of *env-genomic* products, the actual product size was 22 base pairs larger than the genome locations would indicate because of the addition of 11 base pairs on each primer which included an Eco RI restriction enzyme site. C) The single-spliced transcript encoding *env* and vpu products is indicated with the dotted line indicating the intron which is removed by splicing. The nested *env-genomic* primers are indicated, as well as the *env-spliced* primers. D) The multiple-spliced transcripts are represented by a schematic showing one of the *tat* mRNAs. In each case, the first round of PCR was performed with primers S1 and S11 shown in the 5' and 3' most exons. The second round of PCR was performed with primers S4B (for *tat* mRNAs) or S7B (for *nef* mRNAs) which span the first splice site and with primer LA41 found in the 3'most exon. The predicted nested PCR product sizes for *tat* and *nef* transcripts are underlined and indicated to the right. E) Reaction conditions for PCR are listed.



E.

		Mg^{+2} Conc	Annealing Temp	Number of cycles
gag	1st Round 2nd Round	7mM 3mM	47°C 53°C	24 29
pol	1st Round 2nd Round	7mM 3mM	41°C 41°C	24 34
env-genomic	1st Round 2nd Round	4mM 3mM	48°C 50°C	29 30
env-spliced		2.5mM	53°C	29
tat	1st Round 2nd Round	3mM 3mM	52°C 52°C	35 35
nef	1st Round 2nd Round	3mM 3mM	52°C 52°C	35 35
nef-sequence	1st Round 2nd Round	3mM 3mM	56°C 56°C	35 35
β-actin		2.2mM	45°C	29

Samples of patient cells, frozen as described above, were thawed, and the number of viable cells counted in the presence of trypan blue (>95% viable). Aliquots of 5 x 10^6 cells were used for isolation of poly A^+ RNA according to the Micro-Fast Track procedure (Invitrogen, San Diego, CA.). The purified polyA + RNA was treated with RNase-free DNase I (Boehringer Mannheim, Gonbt, Germany, 40 units) at 37° C for 10 min in the presence of 1 ug of carrier tRNA, 10 mM $MgCl_2$, 0.1 mM dithiotheritol and 200 U RNasin (Promega, Madison, WI). Each reaction was stopped by addition of 4 ul of 0.5 M EDTA and 4 ul of 10% SDS. DNase-treated polyA + RNA was extracted once with phenol:chloroform:isoamyl alcohol (100:96:4) and the aqueous phase was precipitated by the addition of glycogen carrier (10 ul), 30 ul of 2 M sodium acetate and 3 volumes of cold ethanol and kept at -70° C overnight. The precipitate was collected by centrifugation, dried at room temperature and dissolved in elution buffer.

Quantitation of polyA + RNA was accomplished by measuring the incorporation of oligo-dT-directed incorporation in the presence of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) of ^{3}H-dCTP into DE81-retainable material, using purified globin mRNA (Gibco/BRL/Life Technologies, Gaithersburg, MD) as a standard. A 2 ul sample of each purified RNA from above was denatured at 65 C for 3 min and then added to a reaction mixture (final volume 20 ul) containing the following: 80 U RNasin, 0.02 OD of oligo $p(dT)_{12-18}$, 0.4 mM each of dATP, dGTP and dTTP, 5 uCi ^{3}H-dCTP (28 Ci/mmole; ICN Radiochemicals, Irvine, CA), 50 mM Tris-Cl (pH 8.3), 30 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol and 20 U of M-MuLV RT (Gibco/BRL, Gaithersburg, MD). The reaction was incubated at 37° C for 1 hr and stopped by the addition of 4 volumes of 10 mM Tris-Cl (pH 7.5) containing 1 mM EDTA. Duplicate 50 ul samples were spotted onto DE81 filter paper discs and dried. Filters were washed 5 times in 5% sodium phosphate dibasic, once in 95% ethanol, dried and counted in a liquid scintillation counter. A standard curve using 50 pg to 2.5 ng of purified globin mRNA was run with each assay and was used to calculate RNA concentrations of patient samples. Samples that did not fall within the linear portion of the standard curve were diluted appropriately and the assay repeated.

<u>Reverse Transcrip</u> <u>:-Polymerase Chain Reaction (RT-PCR)</u>

The RT reaction was performed using 1.5 x 10^5 cell equivalents of RNA in a final volume of 20 ul containing the following: 1 mM each of dATP, dCTP, dGTP and dTTP, 6.5 mM $MgCl_2$, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 20 U RNasin, 2.5 uM random primers (Gibco/BRL) and 2.5 U of M-MuLV RT (Gibco/BRL). The reaction was incubated at room temperature for 10 min and then at 42° C for 35 min and was terminated by heating at 99 C for 5 min.

For PCR, the 20 ul reaction from above was added to a final volume of 50 ul containing the following: 400 uM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 0.01% gelatin, 10 pmoles of each primer, 1.25 units of Taq polymerase (Amplitaq, Cetus Corp., Emeryville, CA) and $MgCl_2$ as shown in Fig. 1E. Primers, annealing temperatures, cycle numbers and expected product sizes for PCR amplifications are shown in Fig 1B-E. The genomic locations of all JA primers were given by Albert and Fenyo.[15] The env primer set (JA9E, JA10E, JA11E and JA12E) are as described by Albert and Fenyo with the addition of an EcoRI site at the 5' end of each primer. SK primers were as described by Ou et al.[16] Beta-actin primers were described by Michael et al.[17] The sequences of other primers are as follows:

S1:	5'-TCTCTCGACGCAGGACTCGGCTTGC-3'	HIV-1 nucleotides: 226- 250
Senv:	5'-CCACACAACTATTGCTATTATT-3'	HIV-1 nucleotides: 5707-5686
S11:	5'-TCCAGTCCCCCCTTTTCTTTTAAAAA-3'	HIV-1 nucleotides: 8641-8666
S4B:	5'-GAGGGGCGGCGACTGAATTGGGTGTC-3'	HIV-1 nucleotides: 275-287, 5357-5368
S7B:	5'-GGGGCGGCGACTGGAAGAAGCGGAGA-3'	HIV-1 nucleotides: 275-287, 5557-5569
LA41v:	5'-CTTCGGGCCTGTCGGGTCCCCTCGGG-3'	HIV-1 nucleotides: 7995-7970
NefAS:	5'-AGCACTCAAGGCAAGCTTTATTGAG-3'	HIV-1 nucleotides: 9210-9186
8306:	5'-TTCCGAATTCAAGCTTGTAGAGCTATTCGCC-3'	HIV-1 nucleotides: 8306-8325
9134:	5'-TTCCGAATTCGAGCTCCCAGGCTCAGATCTGG-3'	HIV-1 nucleotides: 9134-9113

The initial RT-PCR assay on a particular RNA preparation included reactions with or without the RT, as a control. Only samples that yielded no product in the absence of RT, thus indicating the complete removal of DNA, were used for further analysis. DNA products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Quantitation of HIV-Specific mRNAs by PCR

Quantitation of HIV envelope-specific mRNA was accomplished using essentially the same reaction mixtures as above except that 9 pmoles of unlabeled
S1 primer was coupled with 1 pmole of ^{32}P end-labeled Senv primer (3 x 10^6 cpm/pmole) for the amplification reaction. The amplification was carried out for 29 cycles. Products were subjected to electrophoresis in 8% polyacrylamide gels. Gels were dried and exposed to Kodak XAR film with intensifying screens at -75° C for 5 days and scanned with an LKB laser densitometer.

Quantitation of HIV nef-specific mRNA was carried out by performing PCR on two-fold serial dilutions of the RNA. RNA was diluted with a solution of purified yeast tRNA (4 ug/ml), 5 mM DTT and 1 U/ul of RNasin.

Sensitivity of tat and nef PCR

The plasmid pCV1, containing a 1.8 kb insert of a cDNA copy of tat mRNA, and the plasmid pCV3, containing a 1.7 kb insert of a cDNA copy of nef mRNA,[9] were used to determine the sensitivity of the *tat* and *nef* nested PCR reactions using DNA as a target. S1 and S11 were used in both cases as the outside primers (Fig. 1D). With pCV1, at least 7 copies of *tat* DNA could be detected in a reaction mixture when S4B and La41V were used as the inside primers. With pCV3 DNA as a target and S7B and La41V as inside primers, at least 7 copies of DNA could be detected by PCR.

The sensitivity of the nested PCR reaction for detection of *tat* and *nef* RNA transcripts was also determined. pCV1 and pCV3 were digested with PstI and cloned into Bluescript KS (Pharmacia LKB Biotechnology, Piscataway, NJ). Bluescript plasmids were linearized using SmaI for the *tat* plasmid or EcoRI for the *nef* plasmid. *In vitro* transcription products were synthesized with T3 or T7 polymerase according to a standard protocol (Promega) using an *in vitro* transcription kit (Stratagene, LaJolla, CA). RNA products were treated with DNase prior to analysis on ethidium bromide-stained formaldehyde gels. RNA was purified through two push columns (Stratagene) and the optical density of the final product was determined and used to calculate RNA copy number for PCR sensitivity analysis. Our nested primer method could consistently detect 10 to 100 copies of *in vitro* synthesized transcripts.

RESULTS

Immunologic characteristics of study patients.

Blood samples from the 53 patients were analysed for immunologic markers. CD4+ lymphocyte counts ranged from 0 to 1280 per mm^3 (mean = 352 per mm^3; normal range 1000-1500 per mm^3); CD4% ranged from 0 to 49% (mean = 20%); CD8+ cell counts ranged from 37 to 2065 per mm^3 (mean = 924 per mm^3); CD8% ranged from 22 to 85 (mean = 55%); and CD4/CD8 ratio ranged from 0.16 to 1.81 (mean = 0.21). The subjects are listed in Table 1 according to increasing number of CD4+ lymphocyte counts.

Strategy for the detection of HIV RNAs

As shown in Figure 1, HIV RNAs were detected with nested sets of PCR primers. The locations of primers within the HIV genome and relative to each other are shown in Figure 1B-D. Primer pairs for *gag* and *pol* were designed to amplify unspliced RNAs (Fig. 1B), and primer pairs for *env-spliced* (Fig. 1C), *tat* (Fig. 1D), *nef* (Fig. 1D), and *nef-sequence* (using primers S7B and NefAs in the 1st round and primers 8306 and 9134 in the 2nd round, Fig. 1E) were designed to amplify spliced mRNA. Whereas primers for *env-genomic* are capable of amplifying both unspliced and single-spliced transcripts, primers for *env-spliced* amplify only spliced *env* mRNA. Two different sets of primers were utilized for *nef* mRNA which amplify products of 108 bp (*nef*) or 828 bp (*nef-sequence*). For RNA preparations in which HIV-specific transcripts were not detected, the quality of the RNA was confirmed by RT-PCR with beta-actin primers (Fig. 1E).

Detection of HIV RNAs

The results of the nested PCR assays for HIV RNA with seven different sets of primers are shown in Table 1. Of the 53 patient samples that were analyzed with all seven sets of nested primers, 49 (92.5%) were positive with at least one set of HIV-specific primers. The four samples in which no HIV-1 transcripts were detected were obtained from patients with CD4 cell counts of 383, 454, 587 and 902 per mm^3, respectively. None of the latter four patients had received antiretroviral therapy.

When nested sets of primers were used for the detection of unspliced RNAs, the *gag* primer set yielded 32/53 (60%) positive results, the *pol* nested primer set yielded 34/53 (64%) positive results, and the *env-genomic* nested primer set yielded 32/53 (60%) positive. All of these primer sets should detect unspliced RNA, whereas the *env-genomic* primer set should also detect spliced env RNA species. There was excellent correlation between the results with *gag* and *pol* primer sets, with 26 samples yielding positive results with both primer sets, 6 samples yielding positive results with the *gag* primer set but not the pol primer set, and 8 samples yielding positive results with the *pol* primer set but not the *gag* primer set. Discordances may be due to sequence variation at sites of binding of individual primers. Twenty-two samples were positive with *env-genomic* primers as well as both *gag* and *pol* primer sets, 6 samples positive with *env-genomic* primers and *gag* or *pol* primer sets, 4 samples were positive with *env-genomic* primers but neither *gag* or *pol* primers, and 12 samples negative with *env-genomic* primers were positive with *gag* and/or *pol* primers.

When nested sets of primers were used for the detection of spliced RNAs, the *env-spliced* primer set yielded 23/53 (43%) positive results, the *tat* set yielded 4/53 (7.5%) positive results, nef primers yielded 28/53 (53%) positive results, and the *nef-sequence* primer set yielded 9/53 (17%) positive results. Discrepancies between the two different *nef* primer sets may be due to sequence heterogeneity, as well as the lower sensitivity of the *nef-sequence* primer set compared to the *nef* primer set.

Kendall Tau b correlation coefficients and P-values of significant correlations were determined between results of transcript detection and immunological parameters. Table 2 shows the results of these calculations for transcripts that either approached or attained statistical significance when correlated with various immunologic parameters. Statistical significance (at the level of $p = 0.05$) was achieved in the correlation of *gag* transcripts with CD4%, *env-genomic* transcripts with CD4 cell count, CD4%, and CD4/CD8 ratio, and *nef-sequence* transcripts with CD8%.

Quantitation of HIV RNAs

For 13 of the 23 RNA samples positive with the *env-spliced* primers, sufficient RNA was available for quantitation (Table 1). The CD4 cell counts of the specimens tested ranged from 0 cells/mm^3 to 822 cells/mm^3 . The quantitataive results did not show a significant correlation with CD4 cell count, CD4 percent, CD8 cell count, CD8 percent or CD4/CD8 ratio (data not shown).

For 16 of the 18 RNA samples (from the first 29 samples obtained) which were positive with the *nef* primer set, sufficient RNA was available for quantitation. CD4 cell counts ranged from 1 cell/mm^3 to 822 cells/mm^3 in the samples tested. Again, quantitative results did not show a significant correlation with CD4 cell count, CD4%, CD8 cell count, CD8% or CD4/CD8 ratio (data not shown).

TABLE 2

Kendall Tau b Correlation Analysis Between Presence of HIV-1 RNA Transcripts and Immunologic Parameters

	Correlation Coefficient (P)				
	CD4	CD4%	CD8	CD8%	CD4/CD8
gag	-0.21 (0.06)	-0.23 (0.04)	-0.12 (0.29)	0.05 (0.68)	-0.22 (0.06)
pol	-0.13 (0.27)	-0.22 (0.06)	0.04 (0.75)	0.09 (0.43)	-0.19 (0.09)
env-gen	-0.22 (0.05)	-0.28 (0.02)	-0.06 (0.61)	0.21 (0.07)	-0.27 (0.02)
nef	0.04 (0.74)	0.006 (0.96)	0.20 (0.07)	0.10 (0.41)	0.03 (0.82)
nef-seq	-0.04 (0.74)	-0.04 (0.76)	0.17 (0.14)	0.27 (0.02)	-0.06 (0.61)

(7) CONCLUSIONS

Our results support and extend the conclusions of other investigators who have documented the presence of HIV-1 transcripts at all stages of disease. Thus, we and others have detected mRNA for structural proteins in a high percentage of patients, and at all levels of CD4 lymphocytes.[6,18,19] In addition, our study is the first to examine the expression of *tat* and *nef* mRNAs in a cross-sectional cohort of subjects. We have shown that mRNAs for these regulatory proteins are also present in peripheral blood mononuclear cells of patients with CD4 cell counts in the range of 0 to 822 cells per mm^3. Whereas *tat* mRNA is rarely detectable under the current assay conditions, *nef* mRNA is detectable in the majority of HIV-1 infected individuals. The higher level of *nef* than *tat* transcripts may be a reflection of similar results obtained in studies of HIV-1 infection *in vitro*.[20-22]

Schnittman et al. used an RT-PCR technique to assess the transcriptional activity of HIV-1 in peripheral blood mononuclear cells from infected patients.[6] They found viral transcripts in 84% of the individuals, regardless of the clinical status of their HIV disease or the use of antiretroviral therapies. In addition, they showed a significant correlation between the presence of *gag* mRNA and CD4 counts of less than 30%. This finding is confirmed in the present study, and extended by demonstrating that *env-genomic* transcripts were also significantly associated with depressed CD4 cell count and CD4/CD8 ratio. It remains to be determined by longitudinal studies whether the presence of any of these transcripts in the peripheral blood mononuclear cells of a single patient is predictive of more rapid progression or more severe disease.

Schnittman et al also detected spliced mRNAs containing the major splice junction (msj) and tat/rev.[6] Only 6 of 49 patients had detectable msj transcripts whereas 29 of 49 had tat/rev transcripts but no significant correlations with other parameters were demonstrated. We detected *tat* mRNA in only 4 of 53 patients, *nef* was detected in 28 of 53 and using *nef-sequence* primers we detected *nef* RNA in 9 of 53. There was a significant ($p=0.02$) correlation of CD8% with the presence of transcripts detectable with the *nef-sequence* primer set. The significance of this finding with respect to disease stage or progression is not certain at present. The ability of these PCR techniques to detect unspliced and spliced HIV RNAs in patient PBMCs at all stages of disease demonstrates the breadth of transcriptional activity of HIV and underscores the contention that true latency does not exist in this infection.

Michael et al. have recently demonstrated that progression of HIV disease is characterized by a conversion of transcripts from spliced to unspliced genomic RNA.[17] Seshama and colleagues also reported higher ratios of levels of spliced and unspliced mRNAs in asymptomatic HIV-1 infected individuals compared to AIDS patients.[23] These findings reflect the findings obtained with an *in vitro* model of HIV-1 latency which shows a preponderance of multiple-spliced over unspliced transcripts. When HIV-1 is activated from latency *in vitro*, the ratio of unspliced to multiple-spliced transcripts decreases.[24] Although most of our data are not quantitative, the significant inverse correlations of the presence of *gag* and *env-genomic* transcripts with CD4 number and percentage support these observations.

The current study is limited to examining cellular RNAs rather than free genomic RNA in the plasma. Ottmann and colleagues demonstrated HIV-1 genomic RNA in 95% of infected individuals,[25] and Bagnarelli and colleagues show the levels of plasma RNA levels correlated with disease stage better than did the levels of cell-associated RNAs.[26] The current study is also limited to examining viral transcripts in PBMCs which may differ from mRNAs present in tissues. Analysis of viral transcription *in vivo* should provide further understanding of HIV-1 pathogenesis, and may serve as a marker for monitoring antiviral therapies.

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(9) APPENDIX

Cumulative List of Peer Review Publications Supported by this Contract

Arens M, Josephs T, Nag S, Miller JP, and Ratner L. Spliced and unspliced mRNAs for regulatory and structural genes of HIV-1 are detectable at all levels of CD4 cell counts. AIDS Res. & Hum. Retrovir., Submitted.

McNearney T, Hornickova Z, Markham R, Birdwell A, Arens M, Saah A, and Ratner L. Relationship of HIV-1 sequence heterogeneity to stage of disease. Proc. Natl. Acad. Sci. U.S.A. 89:10247-10251, 1993.

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Cumulative List of Reviews Supported by this Contract

Bryant M and Ratner L. The biology and molecular biology of HIV. Ped. Infect. Dis. J., 11:390-400, 1992.

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Nag S, Joseph T, Ratner L, and Arens M. Association of envelope gene expression with HIV-1 disease progression in vivo. Cold Spring Harbor RNA Tumor Virus Meeting, 1992.

McNearney T, Hornickova Z, Arens M, Markham R, and Ratner L. Heterogneeity of the immunodominant V3 loop of HIV-1 envelope sequentially derived from an adult. American Society of Virology, 10th Annual Meeting, Fort Collins, Colorado, 1991.

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